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Changes in the Distribution of Hepatic Copper in Relation to the Progression of Wilson's Disease (Hepatolenticular Degeneration)

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WILSON'S DISEASE is the result of a genetic abnormality leading to the accumulation of excessive amounts of copper, which in time causes pathologic changes in the patient's liver, brain, kidney, and cornea.[1-3] Deficiency or absence of serum ceruloplasmin, together with elevated concentrations of hepatic copper, are both characteristic and diagnostic abnormalities which precede the appearance of histopathologic hepatic changes.[4] In young, asymptomatic patients diagnosed by these findings, the first recognizable histopathologic change consists of fatty metamorphosis, followed by either fibrosis or necrosis of parenchymal cells, ultimately progressing to postnecrotic cirrhosis.[4-7] In contrast, in normal newborns, the physiologic deficiency of serum ceruloplasmin[8] and high concentrations of hepatic copper[9] present during the first few months of life are not followed by these pathologic changes. We were, therefore, led to compare the cytochemical distribu-

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Table 1. Clinical Data, Duration of Treatment, and Histologic Findings in Patients with Wilson's Disease

Patient No.*	Age (yr.)	Sex	Liver Cu (μg./gm. dry liver)	Serum ceruloplasmin (mg./100 ml.)	D-Penicillamine therapy (yr.)	Clinical status	Type of biopsy†	Light microscopic results	Copper staining
1	3½	M	1571	1.0	0	Asymp.	N	Fatty change, moderate	Generalized cytoplasmic, 4+
2 (7)	3½	M	1092	1.0	0‡	Asymp.	W	No changes	Generalized cytoplasmic, 3+
3 (6)	5	F	1644	1.0	2§	Asymp.	N	Fatty change, mild	Generalized cytoplasmic, 4+
4 (11)	5	M	1340	1.0	0	Asymp.	N	Fibrosis; fatty change	Generalized cytoplasmic, 2+
5	10	F	1004	8.4	0	Asymp.	N	Fibrosis; fatty change, severe	Generalized cytoplasmic, 2+
6	11	F	—	5.0	0	Hepatic decomp.	W	Active, nodular cirrhosis	Cytoplasmic & lysosomes‖
7	11	F	892	3.0	0	Hepatic decomp.	W	Active, nodular cirrhosis	Cytoplasmic & lysosomes
8	11	F	507	7.1	0	K-F rings¶	W	Nodular cirrhosis	Cytoplasmic & lysosomes
9	36	M	1088	1.0	0	Neurol. & hepatic	W	Nodular cirrhosis; *Schistosome* granuloma	Cytoplasmic & lysosomes
10	16	M	687	5.9	0	Neurol. & hepatic	N	Early cirrhosis	Cytoplasmic & lysosomes
11	18	M	576	5.3	0	K-F rings	N	Fibrosis	Lysosomes
12	18	M	392	1.0	0	Neurol. & hematol.	N	Nodular cirrhosis	Lysosomes
13	27	M	241	1.0	1	Neurol. & psychiat.	N	Fibrosis; fatty change, minimal	Lysosomes
14	51	F	471	4.3	1	Neurol.	N	Nodular cirrhosis	Lysosomes‖
15	10	M	832	1.0	0‡	Asymp.	N	Fibrosis; fatty change, severe	No staining
16 (31)	17	F	778	1.0	6	Asymp.	N	Fatty change, mild	No staining
Normal			20–45	20–40					

* Numbers in parentheses refer to Case No. in Reference 4.
† N, needle biopsy; W, surgical wedge biopsy.
‡ Low copper diet for preceding 2½ years (Patient 2) and 6 months (Patient 15).
§ Regimen followed erratically during year preceding biopsy.
‖ A few granules that stained for iron were present in specimen.
¶ Kayser-Fleischer rings.

tion of copper and the morphologic and ultrastructural characteristics of hepatic biopsy specimens obtained during various stages of Wilson's disease with the parallel findings in normal neonatal liver. We used both rubeanic acid, a standard cytochemical technique for demonstration of copper,[10,11] and a recently introduced silver sulfide procedure for staining heavy metals.[12,13] This last procedure could be interpreted as demonstrating copper in liver cells, provided they were free of stainable iron. In some specimens, results of the cytochemical findings were correlated with results of electron probe microanalysis and ultrastructural studies.

Materials and Methods

Patients with Wilson's Disease

A total of 16 subjects with Wilson's disease constituted our clinical material. Of these, 9 manifested signs and symptoms of Wilson's disease and 7 were asymptomatic.[4] Each patient exhibited a deficiency of serum ceruloplasmin and an increased hepatic copper concentration, the latter determined on a biopsy sample which was also examined histologically. Four patients had been treated with a low-copper diet and the daily, continued administration of D-penicillamine (Cuprimine, Merck Sharp & Dohme) in doses of 1–3 gm. daily.[14] Clinical data, duration of treatment, and histologic findings are summarized in Table 1.

Specimens of Newborn Liver

Necropsy specimens from 4 premature infants who died of the idiopathic respiratory distress syndrome (hyaline membrane disease) were fixed in phosphate-buffered formalin from 3 to 21 hr. after death and were stored in the fixative for 2–5 months. Birth weights and copper assays are listed in Table 2.

Table 2. Newborn Liver Copper Levels

Patient	Birth wt. (gm.)	Gestational age (estimated wk.)	Copper content (μg./gm. dry liver)
A	600	25	381
B	1400	30	310
C	1300	30	218
D	1460	30	202

Specimens of Control Liver

Four needle biopsy specimens from patients with postnecrotic cirrhosis and three from normal subjects were also stained. One of the latter subjects was a heterozygous sister of a patient with Wilson's disease. She exhibited a serum ceruloplasmin of 24 mg./100 ml. and normal hepatic copper concentration (30 μg./gm. dry liver).

The biopsy specimens of liver were obtained by wedge resection at laparotomy (5 patients) or with the aid of the Menghini needle (11 patients and 7 controls) either for diagnostic purposes (Patients 1, 2, 4–12, and 15, and all 7 controls) or as a guide to continued therapy with D-penicillamine (Patients 3, 13, 14, and 16).

Concentrations of ceruloplasmin were measured by the oxidase activity of serum toward paraphenylenediamine.[15] Hepatic copper was determined by using a modification of a spectrophotometric method after wet digestion of the specimens.[16]

Light Microscopy

Staining for Copper. The 3-μ sections of tissues that had been fixed in formalin or glutaraldehyde and embedded in paraffin were brought to water and immediately stained by a slight modification [17] of Timm's silver sulfide technique.[12,13] The three solutions used were: (1) water saturated with hydrogen sulfide, (2) 5% silver nitrate, and (3) 2 gm. hydroquinone and 5 gm. citric acid in 100 ml. water. Slides were placed in a covered jar containing Solution 1 for 15 min., carefully rinsed, and then flooded for 90 sec. with a solution consisting of 1 part of Solution 2 and 5 parts of Solution 3. They were rinsed again and mounted in glycerine jelly.

Specimens were also stained with rubeanic acid (dithiooxamide, Eastman Organic Chemicals). Frozen sections or paraffin-embedded sections were brought to water and stained for 72 hr. at 37° C. in a medium prepared by adding, after filtration, 5 ml. of a saturated solution of rubeanic acid in absolute ethanol (approximately 2%) to 100 ml. of a 10% solution of sodium acetate in distilled water. Sections were washed and mounted in glycerine jelly, or dehydrated and mounted in Permount.

Staining for Iron. All specimens were stained by Perls's ferrocyanide and Quincke's iron sulfide procedures.[18]

Enzyme Cytochemistry. For demonstration of lysosomes, freely floating 10-μ frozen sections of five specimens (Patients 5, 11–13, and 15) that had been fixed in a cold 3% solution of glutaraldehyde in cacodylate buffer pH 7.4 [19] for 3 hr. were incubated for: (1) acid phosphatase activity by Gomori's technique [18] with β-glycerophosphate as substrate, and in Barka and Anderson's hexazonium-pararosaniline medium with naphthol AS-TR phosphatase as substrate; [20] (2) β-glucuronidase activity in the medium of Hayashi, Nakajima, and Fishman; [21] and (3) N-acetyl β-glucosaminidase activity in Hayashi's medium.[22]

Electron Microscopy

Staining for Copper. The 30-μ frozen sections of glutaraldehyde-fixed tissue from three liver biopsy specimens from patients with Wilson's disease (Patients 5, 11, and 12) and one formalin-fixed specimen from the liver of a premature infant were stained by the silver sulfide technique, rinsed in a 0.25 M sucrose solution, postfixed for 45 min. in 1% osmium tetroxide in phosphate buffer (0.1 M, pH 7.4), and embedded in Epon. Thin sections were examined in an RCA EMU 3-B electron microscope.

Enzyme Cytochemistry. For demonstration of lysosomes, 40-μ frozen sections of two glutaraldehyde-fixed liver biopsy specimens (Patients 11 and 12) were incubated for acid phosphatase activity in Gomori's medium, postfixed in osmium tetroxide as above, and embedded in Epon.

Electron Probe Microanalysis

Sections, 3–4 μ in thickness, of glutaraldehyde-fixed tissue from two specimens (Patients 3 and 11) that had been embedded in Epon, and from one paraffin-embedded specimen (Patient 6), were mounted on glass slides, coated in vacuum with a heavy layer of carbon, and examined with a Norelco AMR-3 electron probe microanalyzer, as previously described.[23] Areas chosen at random were analyzed for copper with a 1-μ beam. The counting and timing circuits were programmed

to accumulate the emitted X-ray impulses from each point for an integration period of 100 sec. These data were used to plot the graphs on Fig. 12.

Results

Both the rubeanic acid and silver sulfide staining procedures demonstrated that in patients with Wilson's disease copper was not always uniformly distributed, and in several biopsy specimens some nodules stained darkly while others showed no copper, confirming earlier studies.[24-29] The intracellular distribution of copper also varied with the age of the patient and the stage of the disease. Copper was (1) diffuse in the cytoplasm of hepatocytes; (2) diffuse in the cytoplasm and concentrated in lysosomes; or (3) found only in lysosomes. Copper in lysosomes was stained by both the rubeanic acid and the silver sulfide techniques, but only the latter procedure visualized diffuse cytoplasmic copper.

Copper Staining

Diffuse Cytoplasmic Copper. In 5 asymptomatic patients (Patients 1–5) with markedly elevated levels of hepatic copper (1004–1644 μg./gm. dry liver), the diffuse pattern of staining was the same in all parts of the specimen (Fig. 1). Under the electron microscope, staining was also diffuse within the cell, without a preferential localization to any organelle (Fig. 5). During this stage of the illness cytoplasmic fat droplets were common, but fibrosis was absent or minimal (Fig. 1 and 3).

The lysosomes in specimens with diffuse copper staining showed normal staining patterns, with acid phosphatase (Fig. 6), β-glucuronidase, and glucosaminidase activities localized to pericanalicular granules.

Diffuse Cytoplasmic and Lysosomal Copper. In five specimens from untreated patients with hepatic or neurologic symptoms of Wilson's disease (Patients 6–10) and with hepatic copper concentrations ranging from 507 to 1088 μg./gm. dry tissue, unstained nodules, as well as nodules with diffuse cytoplasmic staining, and areas in which only lysosomes stained darkly for copper (Fig. 2) were seen. In four of these specimens active liver disease with necrosis, inflammation, regeneration, and nodular cirrhosis were seen as prominent features (Patients 6–9), and in the fifth (Patient 10) there was early cirrhosis.

Lysosomal Staining. In 4 older patients, all with neurologic symptoms and signs of Wilson's disease (Patients 11–14), hepatic copper concentrations ranged from 241 to 576 μg./gm. dry tissue. Comparison

of sections stained for copper with those stained for acid hydrolase activity showed that the same granules contained both. Copper staining (brown-black in silver sulfide preparations, green-black with rubeanic acid) was seen only in granules that resembled lysosomes (Fig. 7), as illustrated in the biopsy specimen from an 18-year-old (Patient 11). Lysosomes in this specimen, visualized by incubation for acid phosphatase (Fig. 8), β-glucuronidase (Fig. 9), and glucosaminidase activities (not illustrated), were often huge, measuring up to 7 μ. In the light microscope it was evident that the granules had the golden brown color of lipofuscin pigment; this was confirmed in specimens from this group of patients examined by electron microscopy (Fig. 10 and 11).

Absence of Staining in Proven Cases. Biopsy specimens from 2 asymptomatic patients (Patients 15 and 16) with fatty changes in hepatocytes, minimal fibrosis, and copper concentrations of 832 and 778 μg./gm. dry liver, respectively, did not stain for copper with either rubeanic acid or the silver sulfide procedures.

Controls. Seven control specimens were entirely free of copper staining (Fig. 4). In one specimen, differential staining indicated the presence of iron rather than copper.

Electron Probe Microanalysis

Copper peaks were recorded in 9 of 10 randomly chosen areas of a specimen displaying diffuse copper staining (Patient 3). In contrast, in a second specimen (Patient 11) with only lysosomal staining, only 4 of 10 recordings for copper were obtained, and these were scattered (Fig. 12). In a third specimen in which both copper staining patterns were present in different nodules (Patient 7), the same correlation of each pattern with electron probe recordings was noted.

Newborn Liver

The four specimens from newborns had hepatic copper levels ranging from 210 to 381 μg./gm. dry tissue (Table 2), about 10 times greater than the normal adult values (20–45 μg./gm.; mean 31.5 ± 6.8 μg./gm.). Granules, which also contained brown pigment, were seen in the cytoplasm of hepatocytes and in some ductular epithelial cells at the periphery of the lobules. The granules were evident in hematoxylin and eosin preparations, but were more easily detected in unstained sections; they stained positively for copper (Fig. 13 and 14). Iron, as well as copper, was present in three of the four specimens. Electron microscopy of the specimen free of stainable iron showed the silver to be essentially restricted to the large polymorphic pigmented granules that resembled

the lipofuscin granules of adult tissues (Fig. 15). Although the delimiting membrane was not well preserved, they probably are lysosomes of the residual body type.

Discussion

Three points may be discussed: the sensitivity and specificity of the staining methods used for the cytochemical demonstration of copper; the localization of copper in hepatocytes at various stages of Wilson's disease; and the distribution of intracellular copper under physiologic and pathologic conditions.

Comparison of Methods

Rubeanic acid is the most commonly used copper stain. Yet, whether as described by Uzman [11] or modified by using formalin- or glutaraldehyde-fixed tissues, the stain does not always demonstrate the metal. In some specimens known to have grossly elevated copper concentrations, particularly from asymptomatic patients, the stain has failed in our hands and in those of other workers.[6,28,30] In contrast, Timm's silver sulfide technique gave positive results in almost all such specimens, and thus appeared to be more sensitive than the rubeanic acid procedure. There were, however, two exceptions to the correlation between the intensity of the stain and the quantitative determination of copper concentration. In two asymptomatic patients (Patients 15 and 16) with hepatic copper concentrations of 832 and 778 μg./gm. dry tissue, neither staining method gave positive results. We assume that in these 2 patients, copper was diffusely distributed in the cytoplasm in concentrations that were below the lower limit of detection by available staining techniques.

Timm's silver sulfide stain is sensitive but not specific, because it stains iron and zinc, as well as copper. In the liver of patients with Wilson's disease, however, iron is the only other metal present in sufficient concentrations to be stained. Therefore only in specimens shown to be free of iron by the specific Perls's Prussian blue and Quincke's iron sulfide stains could we conclude that the silver staining seen with Timm's method represented copper. Quantitative copper assays and electron probe microanalyses confirmed our findings.

Localization of Copper

Our results indicate that the localization of copper in hepatocytes of patients with Wilson's disease varies with the stage of the disease. Early, when fatty changes predominate (Patients 1–5), copper is diffuse

in the cytoplasm of the hepatocytes. When hepatocellular necrosis appears (Patients 6–10), this pattern persists in some nodules, while in others in the same specimen the copper stain is concentrated in granules (Fig. 2). Combinations of staining patterns which may be found side by side in adjacent nodules are characteristic of the intermediate stages of Wilson's disease. Later when fibrosis and cirrhosis are the predominant histopathologic features, the metal is stained mainly within the lysosomes of hepatocytes (Patients 11–14). At this advanced stage, overall tissue copper levels are frequently lower than in the earlier phases of the illness, but copper stains with both the rubeanic acid and silver sulfide techniques are regularly positive because the metal is concentrated within lysosomes rather than spread diffusely in the cytoplasm.

Comparison of Distribution of Physiologic and Pathologic Copper

Correlation of these copper staining patterns with histopathologic observations suggests that the diffuse cytoplasmic distribution leads to cellular damage and necrosis, while copper localized to lysosomes may be less toxic. Evidence for this is found in the less conspicuous cytopathologic changes seen in electron microscopic studies of patients with advanced Wilson's disease,[31] in whom copper is concentrated in lysosomes.[17,23] That the uptake of copper by lysosomes is physiologic is indicated by our observation of this phenomenon in neonatal liver, as well as in rats [32–35] and mice,[36] where experimentally induced hepatic copper deposits generally do not produce necrosis.

Correlation of fragmentary observations on the subcellular distribution of copper [37] with copper staining in patients with Wilson's disease confirms our findings. In one patient with diffuse cytoplasmic copper staining (Patient 2; W.B. in Table 2, Ref. 37), 80% of the hepatic copper was found in the supernatant after centrifugation of the liver homogenate at 100,000 *g* for 40 min. In a patient with copper distributed in both cytoplasm and lysosomes (Patient 8, Fig. 2; J.S. in Table 2, Ref. 37), 59% of copper was in this supernatant; and in a patient with advanced Wilson's disease (N't'n in Table 2, Ref. 37) only 26% of the copper was in the supernatant.

It is relevant to the pathogenesis of Wilson's disease to note the association of diffuse copper, fatty changes, and mitochondrial abnormalities in young patients. This was evident in 2 asymptomatic patients (Patients 1 and 3) whose biopsy specimens were studied with the electron microscope and in whom marked mitochondrial changes [38] were

found. In 2 older patients (Patients 11 and 13) with lysosomal copper, the mitochondria appeared unremarkable, and fatty changes were minimal or absent. The experimental evidence showing that copper can induce swelling of mitochondria [39] and alter mitochondrial respiration in vitro [40,41] is pertinent because of this association of diffuse copper staining with mitochondrial changes in vivo.

Early in the disease, although lysosomes appear normal by cytochemical and morphologic criteria that are currently available, their ability to incorporate copper may be blocked by the excessive amounts of copper in the cytoplasm. It is also possible that the affinity for copper of the sulfhydryl-rich protein present in the cytoplasm [37] of these patients may be greater than that of lysosomes. In contrast, in the patient with advanced disease, in spite of the reduced size of the liver and the presence of portal hypertension, the hepatocytes seem to be protected from the toxic effects of copper by segregation of the metal in lysosomes. At present, the factors that modulate the transition from one pattern of distribution of intracellular copper to the other are still unknown.

Summary

The localization of copper in the hepatocytes of patients with Wilson's disease and of newborn infants was studied by light and electron microscopic staining techniques. Striking differences in staining patterns were observed at different stages of Wilson's disease. In the youngest patients, who were asymptomatic, copper staining was diffuse in the cytoplasm. In slightly older patients, who exhibited early symptoms or signs of the disease, the metal was both distributed diffusely in the cytoplasm of hepatocytes and concentrated in lysosomes in different nodules. In the patients with advanced disease and in normal newborn infants, copper was found exclusively in lysosomes.

In the young patients, diffuse copper staining was associated with pronounced cytopathologic changes. The latter were less marked or minimal in the older patients in whom hepatic copper was concentrated in lysosomes. Comparison of these findings and those of newborn livers suggested that sequestration of copper in lysosomes might protect hepatocytes from the toxic effects of the metal.

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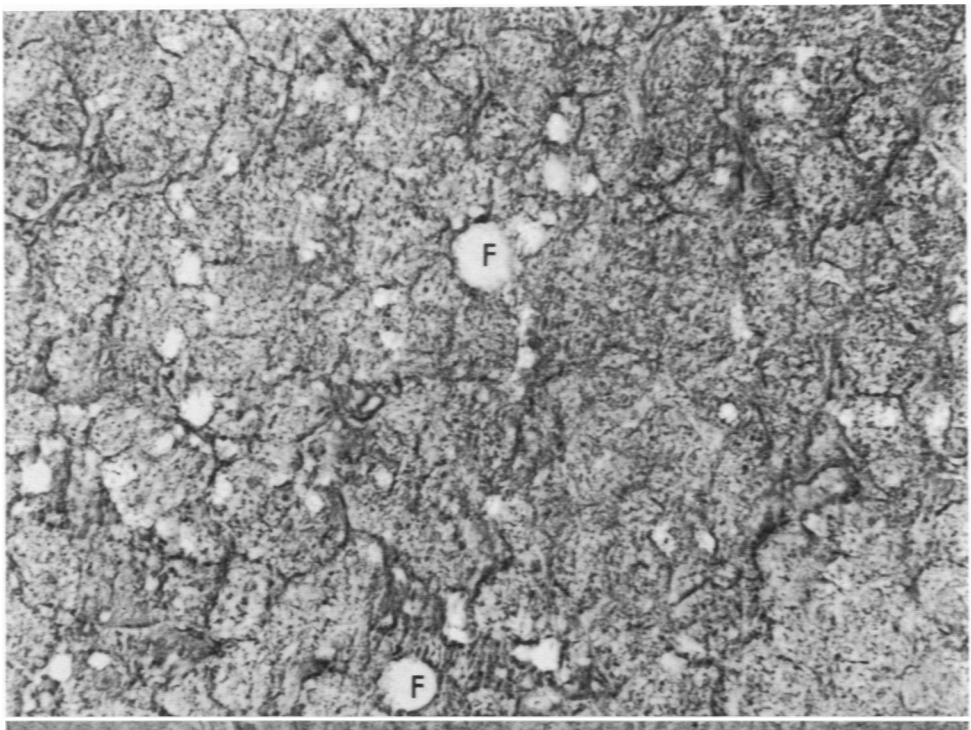
Legends for Figures

Fig. 1. Paraffin section of needle biopsy from 5-year-old girl (Patient 3); stained by silver sulfide procedure. This specimen contained 1644 μg. copper per gram of dry liver. Diffuse, brown, finely granular staining over hepatocytes is uniform throughout entire specimen. Some fatty change is evident *(F)*. × 600.

Fig. 2. Paraffin section of specimen obtained by wedge biopsy from 11-year-old girl (Patient 8); stained by silver sulfide procedure. This specimen contained 507 μg. of copper per gram dry liver. Copper staining varies in different areas: nodule on the left is unstained, and the one on the right is stained diffusely. In tissue at center of field, copper staining is black and localized to coarse cytoplasmic granules. In this part of specimen, hepatic architecture is relatively intact with preserved central veins and portal triads (not shown). In the two other nodules, central veins are absent, cell plates are irregular, and numerous large multinucleated cells are found, suggesting regeneration. × 150.



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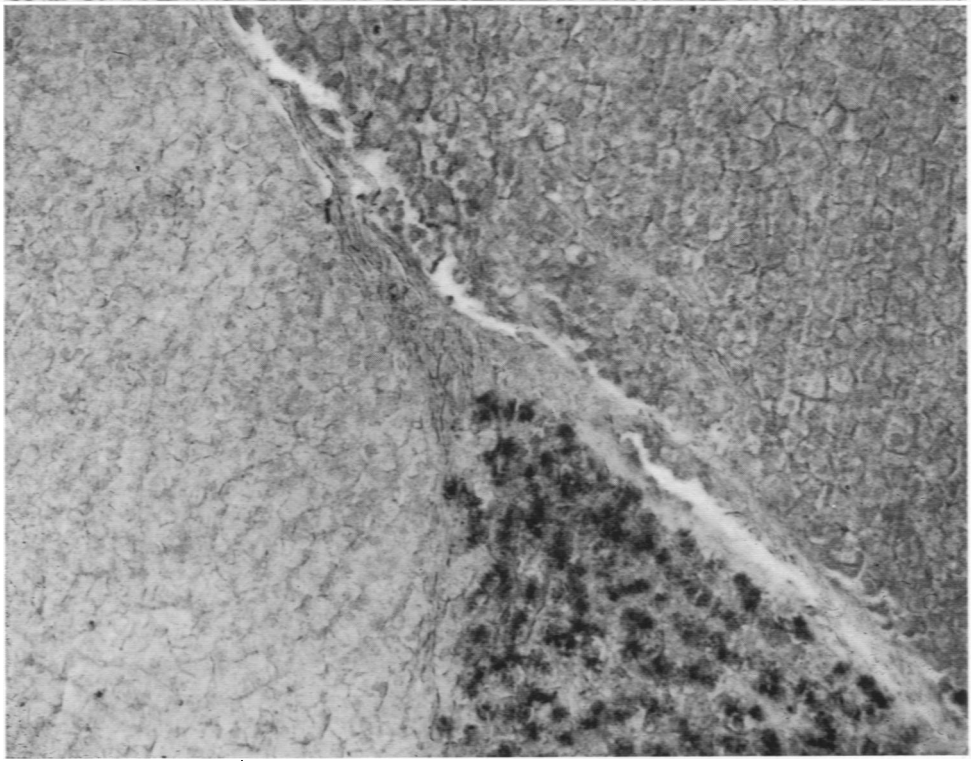

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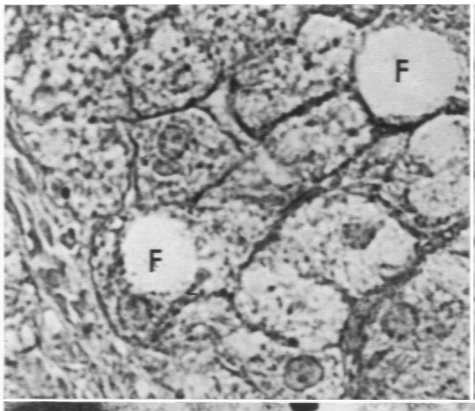
Fig. 3 and 4. Paraffin sections of needle biopsy specimens from two sisters; stained by silver sulfide procedure. Both specimens were prepared and photographed under identical conditions. Fig. 3 and 4 × 500.

Fig. 3. From 10-year-old sister with Wilson's disease (Patient 5). Copper content, 1004 μg./gm. dry liver. Copper staining is diffuse, and fatty change *(F)* prominent.

Fig. 4. From normal sister; copper content, 30 μg./gm. dry liver. No staining seen in this specimen.

Fig. 5. Electron micrograph of 30-μ frozen section of specimen in Fig. 3; stained by silver sulfide procedure. Black granules which represent silver deposits are scattered over cytoplasm and are not restricted to any organelle; *(M)* mitochondrion, *(L)* lysosome. × 38,000.

Fig. 6. A 10-μ frozen section from same specimen as in Fig. 5; incubated for acid phosphatase activity in Gomori's medium. Reaction product is localized to the lysosomes. × 1800.



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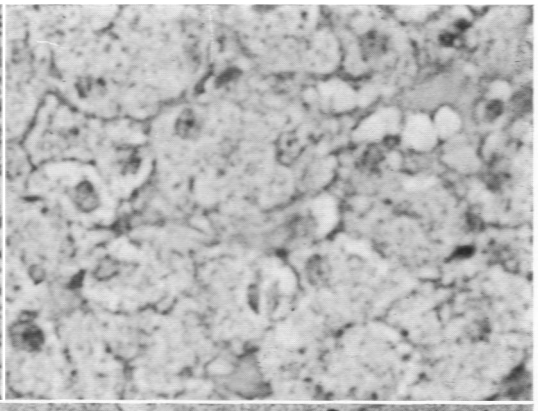

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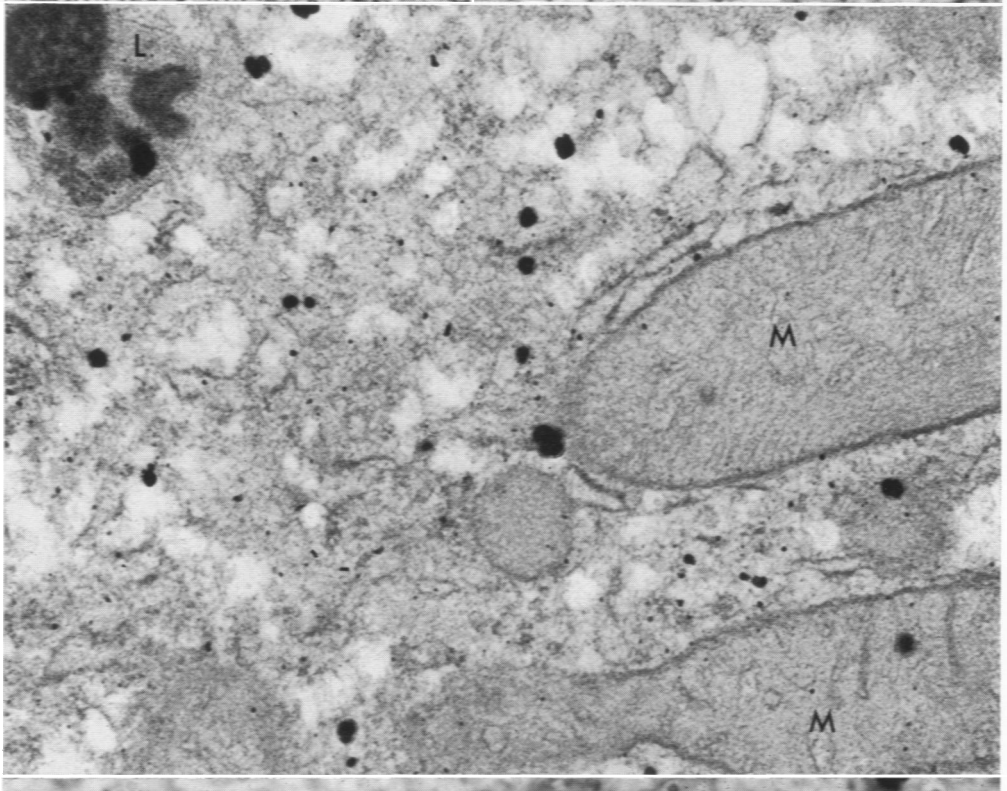

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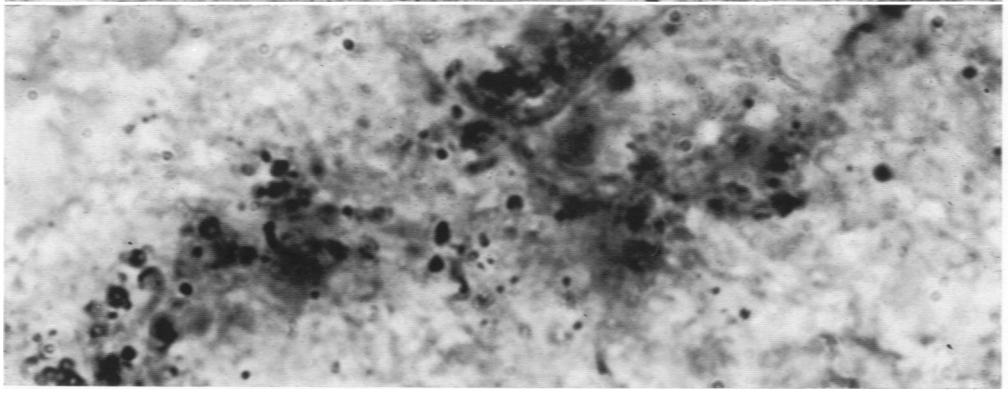

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Fig. 7–9. Needle biopsy specimen from 18-year-old Patient 11 with Wilson's disease; hepatic copper concentration 576 μg./gm. dry tissue.

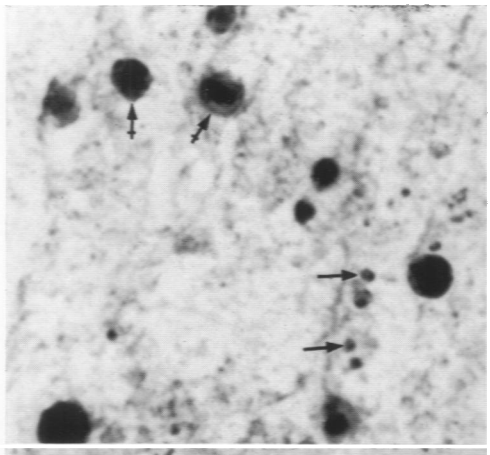
Fig. 7. Paraffin section stained by silver sulfide technique. Stain is localized only to discrete granules within the cytoplasm *(arrows)*. In some areas they are very large: approximately 7 μ in diameter *(crossed arrows)*. × 1200.

Fig. 8. A 10-μ frozen section from same specimen as in Fig. 7; incubated 30 min. for acid phosphatase activity. Enzyme reaction product is present in small pericanalicular granules *(arrows)* and in large granules *(crossed arrows)*, indicating that they are lysosomes. Nucleus *(N)* is unstained. × 1200.

Fig. 9. A 10-μ frozen section from same specimen as in Fig. 7; incubated 45 min. for β-glucuronidase activity. Reaction product of this acid hydrolase is also deposited on small *(arrow)* and large *(crossed arrow)* granules, confirming their identification as lysosomes; *(N)* nucleus. × 1200.

Fig. 10. Electron micrograph of 40-μ frozen section of needle biopsy specimen from 27-year-old man (Patient 13), whose liver contained 241 μg. of copper per gram dry weight; incubated for acid phosphatase activity in a Gomori medium. Enzyme reaction product is deposited on lysosomes *(L)* that have characteristic multilobular structure of lipofuscin pigment granules; *(M)* mitochondrion. × 19,000.

Fig. 11. Electron micrograph of 30-μ frozen section of needle biopsy specimen from 18-year-old man (Patient 12), whose liver contained 392 μg. of copper per gram dry weight; stained by silver sulfide procedure. The silver stain is concentrated in compartments within lipofuscin granules *(L)*. × 33,000.



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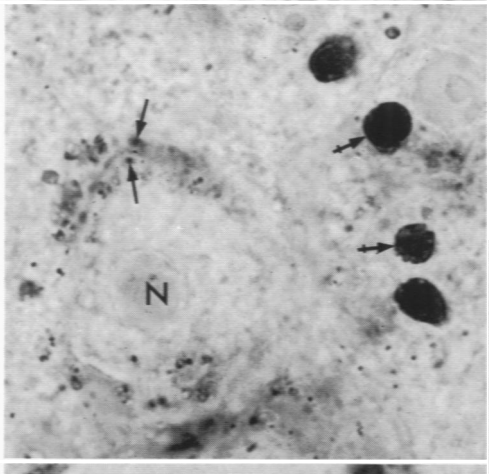

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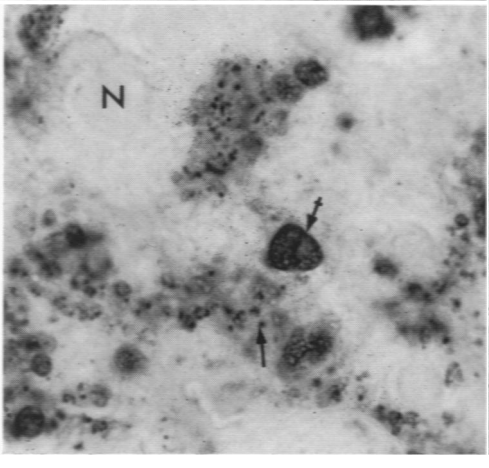

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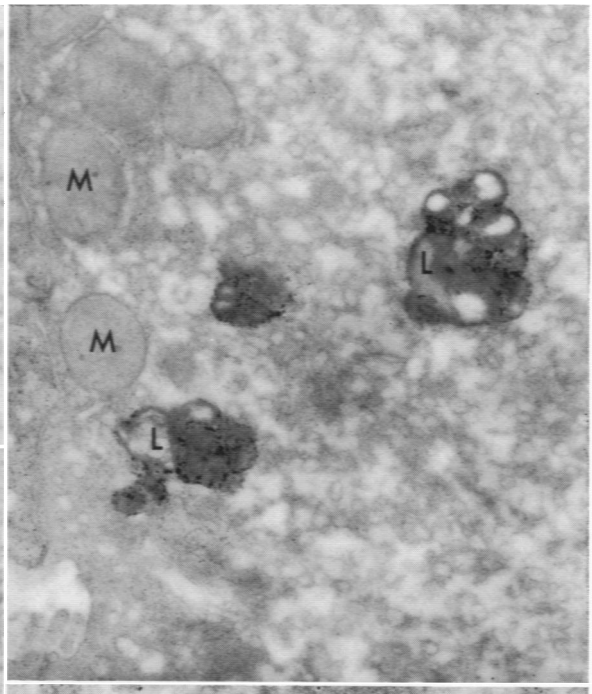

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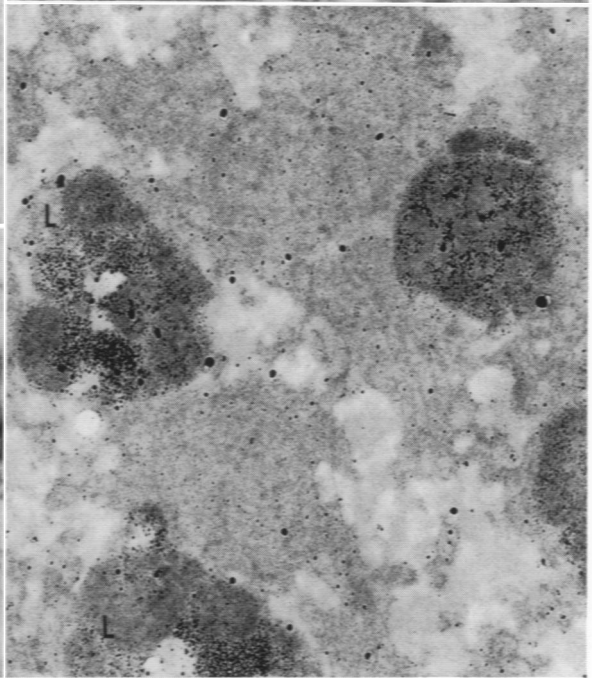

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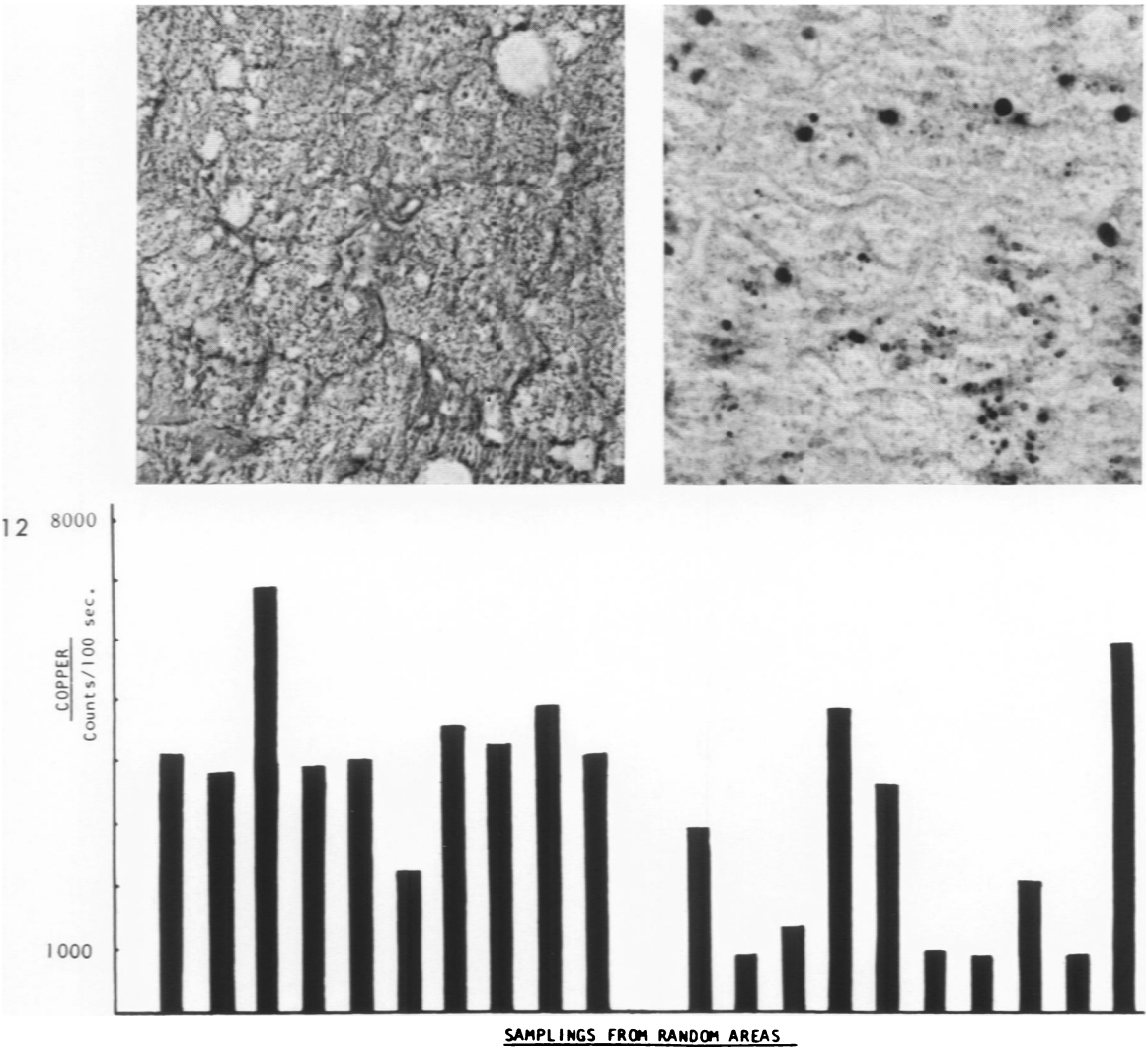
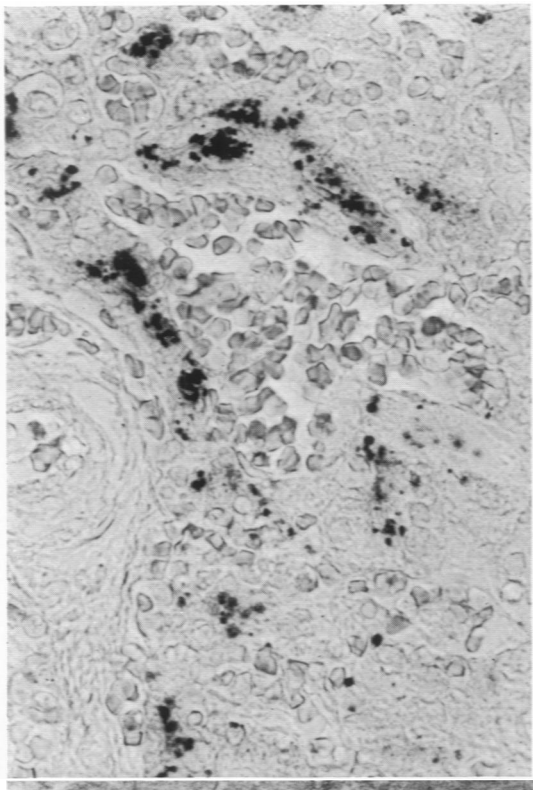


Fig. 12. Electron probe microanalyses recordings of unstained, Epon-embedded tissue from needle biopsy specimens from Patients 3 *(left)* and 11 *(right)*, whose livers showed diffuse and lysosomal copper, respectively. Patient 3: 5 years old; 1644 μg. of copper per gram dry liver. Patient 11: 18 years old; 576 μg. of copper per gram dry liver. In specimen with diffuse staining, 9 of 10 recordings gave significant copper peaks. Only scattered recordings for copper were obtained in the specimen with lysosomal staining. Compare with results of staining for copper in paraffin-embedded sections obtained from the same specimens.

Fig. 13 and 14. Paraffin sections of liver from newborn (Patient B); stained for heavy metals by silver sulfide procedure **(Fig. 13)**, and for copper by rubeanic acid technique **(Fig. 14)**. The metal is localized to granules at periphery of lobule. × 250.

Fig. 15. Electron micrograph of portion of same specimen as in Fig. 13 and 14; stained by silver sulfide procedure. This tissue was obtained 20 hr. post mortem and fixed in formaldehyde for 4 months. Endoplasmic reticulum *(ER)*, mitochondria *(M)*, and a dense body may be identified. The silver stain is localized mainly to a dense, pigmented body which is probably a lysosome. × 13,000.



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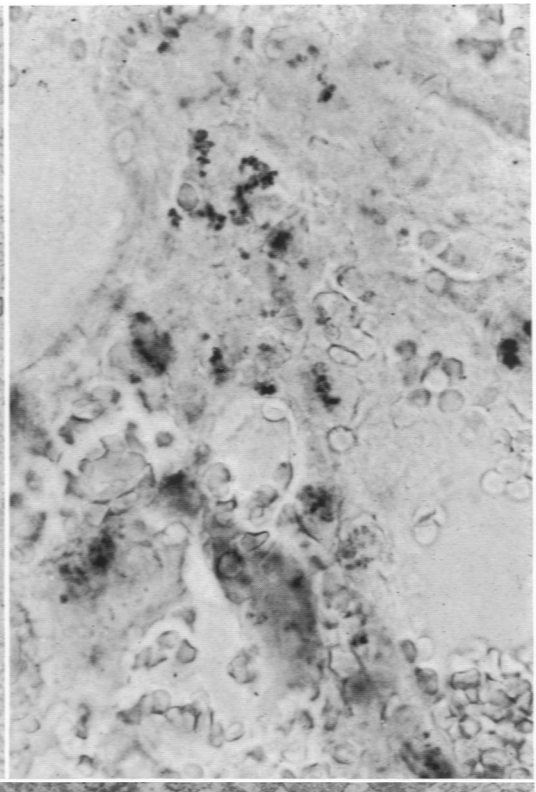

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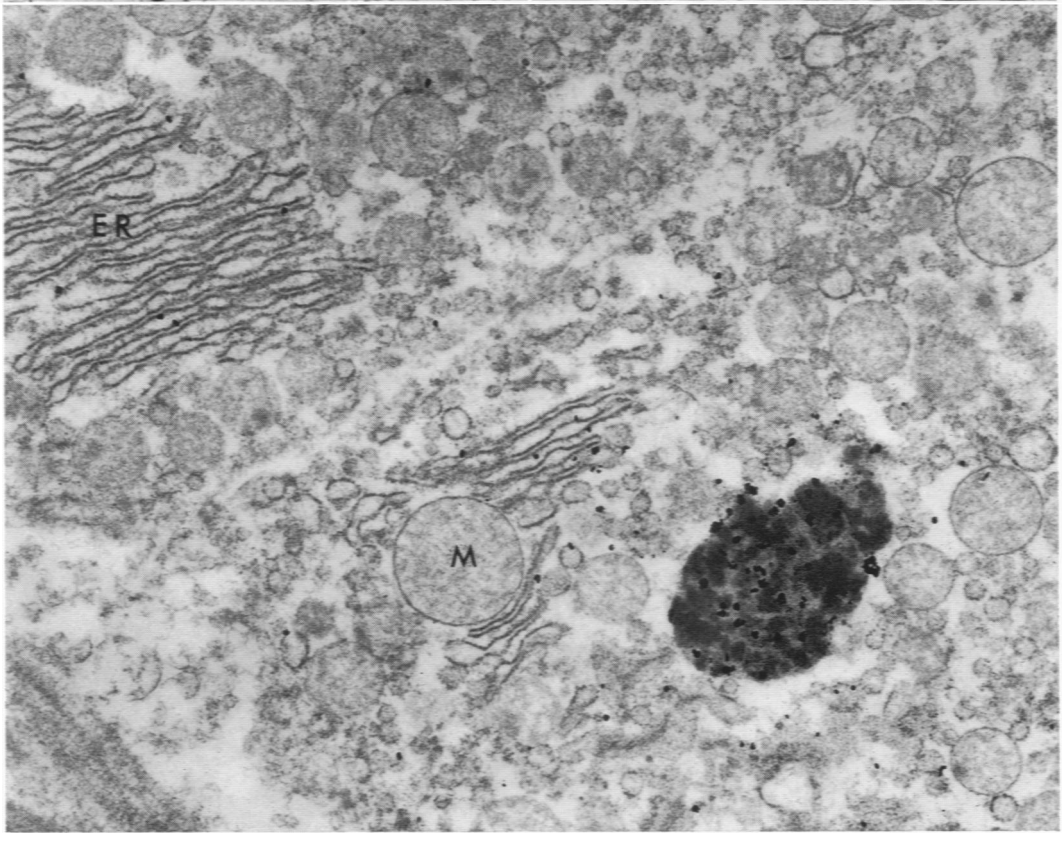

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